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# A sensor for intracellular ionic strength

Esther Biemans-Oldehinkel, Nik A. B. N. Mahmood, and Bert Poolman\*

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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**Cystathionine- $\beta$ -synthase (CBS) domains are found in >4,000 proteins in species from all kingdoms of life, yet their functions are largely unknown. Tandem CBS domains are associated with membrane transport proteins, most notably members of the ATP-binding cassette (ABC) superfamily; voltage-gated chloride channels and transporters; cation efflux systems; and various enzymes, transcription factors, and proteins of unknown function. We now show that tandem CBS domains in the osmoregulatory ABC transporter OpuA are sensors for ionic strength that control the transport activity through an electrostatic switching mechanism. The on/off state of the transporter is determined by the surface charge of the membrane and the internal ionic strength that is sensed by the CBS domains. By modifying the CBS domains, we can control the ionic strength dependence of the transporter: deleting a stretch of C-terminal anionic residues shifts the ionic strength dependence to higher values, whereas deleting the CBS domains makes the system largely independent of ionic strength. We present a model for the gating of membrane transport by ionic strength and propose a new role for CBS domains.**

ATP-binding cassette transporter | cystathionine- $\beta$ -synthase domains | cell volume regulation | ionic strength sensor | osmoregulation

CBS stands for cystathionine  $\beta$ -synthase, the enzyme that catalyzes the condensing of homocysteine and serine and plays a pivotal role in mammalian sulfur metabolism. CBS is composed of a heme-binding, a catalytic, and a regulatory domain (1); the latter domain is a small protein module, known as the “CBS domain.” Point mutations in this domain cause homocystinuria in humans (1), whereas a number of patient-derived mutations in the catalytic domain of the enzyme have consequences that are alleviated by the deletion of the CBS domain (2). The CBS domains are not unique to CBS but can be identified in a wide variety of proteins (see ref. 3 and [www.sanger.ac.uk/Users/agb/CBS/CBS.html](http://www.sanger.ac.uk/Users/agb/CBS/CBS.html)), including ATP-binding cassette (ABC) transporters, voltage-gated chloride channels and transporters, a variety of other transporter families, and a number of enzymes, or exist as tandem domains without catalytic moiety (Table 1, which is published as supporting information on the PNAS web site). Inspection of published genome databases reveals >4,000 proteins that possess CBS domains, in most cases as tandem pairs with a N- $\beta$ - $\alpha$ - $\beta$ - $\alpha$ -C secondary structure (Fig. 7, which is published as supporting information on the PNAS web site). Although proteins with CBS domains are found in all kingdoms of life (Table 1), a regulatory role has been shown in a few cases, and 3D structures of the domains have been determined (4, 5), their regulatory mechanism(s) are unknown. Their importance in biology and medicine is highlighted by the fact that hereditary disease mutations have been found in CBS domains of various proteins (2, 6, 7). Recent work suggests that tandem pairs of CBS domains bind adenine nucleotides or *S*-adenosyl methionine (8). In the case of the skeletal muscle CIC-1 chloride channel, there is evidence that adenine nucleotides affect the gating of the channel (9), but the proposed regulatory role is still enigmatic, because ATP and AMP, but also adenosine, have similar effects.

The ABC transporter OpuA and other types of osmoregulatory transporters (10–12) mediate the uptake of compatible solutes, such as glycine betaine, in response to increasing extra-

cellular osmolality, and thereby reverse the osmotic shrinkage of the cell. When reconstituted in proteoliposomes, the transporters are activated by increased concentrations of luminal ions (10, 13, 14). The activation of the ABC transporter is instantaneous both *in vivo* and *in vitro* and requires only threshold levels of ionic osmolytes. The threshold for activation by ions depends on the ionic lipid content (charge density) of the membrane, indicating that the signal is transduced to the transporter by critical interactions of protein domains with membrane lipids. The ABC transporter OpuA has two CBS domains in tandem at the C-terminal end of the ATPase subunit OpuAA (15). With two ATPase subunits per functional unit, a total of four CBS domains are present in OpuA. The C-terminal tail of the tandem CBS in OpuA is highly charged, with 10 acidic and 2 basic residues in a stretch of 15 amino acids. The charged C terminus of the CBS domains in OpuA and other ABC transporters is unusual, and the length and charge of the tail are highly variable among CBS domains (Fig. 7).

We now show that the activation of ABC transporter OpuA by increased concentrations of luminal ions reflects gating by internal ionic strength. We also demonstrate that the CBS domains of OpuA constitute the lipid-dependent ion-sensing modules of the transporter and describe a specific mechanism for regulation of protein function by these widespread protein domains. The sensing properties of the CBS domains are modulated by the anionic C-terminal tail.

## Results

**Sensing of Ionic Strength.** To investigate the effect of osmotic stress on OpuA from *Lactococcus lactis*, the transporter was reconstituted into liposomes with varying luminal composition, and the activity was determined over a range of external osmolarities. Mg-ATP was present in the vesicle lumen to drive the translocation reaction, and the concentration of potassium phosphate (KPi) was varied from 10 to 120 mM. The concentration of the internal components was isotonic, with 30–140 mM KPi, pH 7.0, in the external medium (0 KCl in Fig. 1A). Fig. 1A shows the activity of OpuA as a function of external osmolality (KCl concentrations ranging from 0 to 300 mM) and varying luminal concentrations of KPi; similar results were obtained when the external osmolality was varied with KPi, NaCl, or sucrose (not shown). Because (proteo)liposomes behave osmotically (water diffuses across the membrane in response to the osmotic difference between the internal compartment and the outside medium), their volume will decrease with increasing concentration of KCl (or any membrane-impermeant osmolyte, e.g., KPi, NaCl, or sucrose) on the outside. The internal osmolality and ionic strength will increase accordingly. The data demonstrate that a larger osmotic upshift was needed to activate OpuA when the internal KPi concentration was lower, irrespective of whether KCl (Fig. 1A) or another ionic or nonionic

Conflict of interest statement: No conflicts declared.

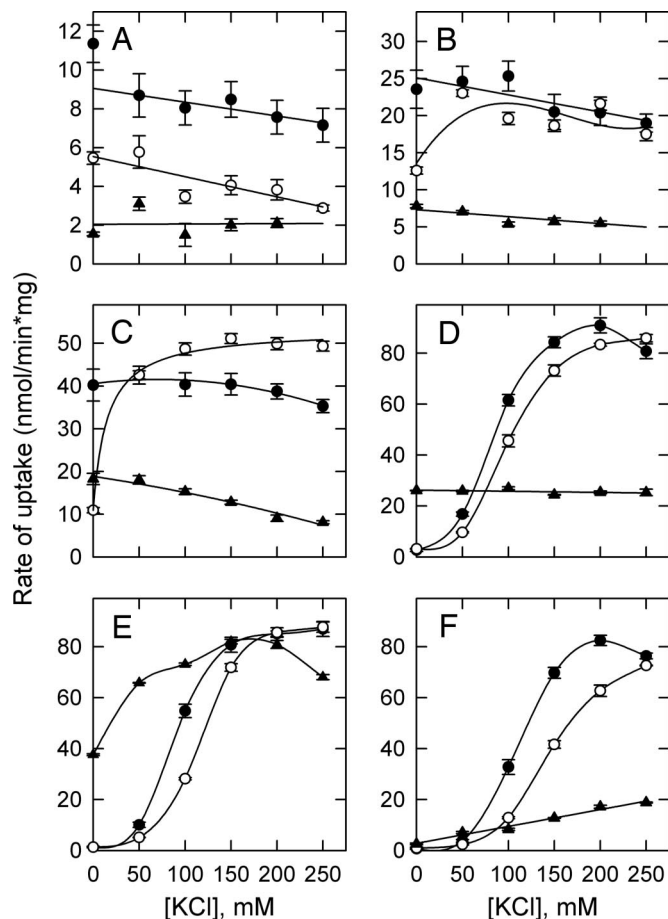
Abbreviations: CBS, cystathionine  $\beta$ -synthase; ABC, ATP-binding cassette;  $I_m$ , internal ionic strength; DOPG, phosphatidylglycerol; DOPE, phosphatidylethanolamine; DOPC, phosphatidylcholine.

\*To whom correspondence should be addressed. E-mail: b.poolman@rug.nl.

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**Fig. 3.** *In vitro* activation profiles of OpuA and derivatives. Uptake of [ $^{14}$ C]glycine betaine by OpuA (●), OpuA $\Delta$ 12 (○), and OpuA $\Delta$ 61 (▲) was assayed in 90 mM KPi, pH 7.0, with or without added KCl as indicated on the x axis. The proteoliposomes were composed of 50 mol % DOPE, 44 mol % DOPC, 6 mol % DOPG (A); 50 mol % DOPE, 37 mol % DOPC, 13 mol % DOPG (B); 50 mol % DOPE, 32 mol % DOPC, 18 mol % DOPG (C); 50 mol % DOPE, 25 mol % DOPC, 25 mol % DOPG (D); 50 mol % DOPE, 12 mol % DOPC, 38 mol % DOPG (E); or 50 mol % DOPE, 50 mol % DOPG (F). The ATP-regenerating system was enclosed inside the proteoliposomes. The average rates and standard deviations of at least four time points in the linear range (10–45 sec) of the uptake curve are shown.

to drive the translocation reaction for prolonged periods of time. With the ATP-regenerating system inside the vesicle lumen, the  $I_{in}$  was  $\approx 0.2$ , and the concentration of the internal components was isotonic with 90 mM of KPi, pH 7.0, in the external medium. By increasing the external KCl concentration from 0 to 0.25 M, the  $I_{in}$  increases from  $\approx 0.2$  to  $\approx 0.7$ . The anionic lipid dependence of OpuA was analyzed by varying the mol % of DOPG from 6 to 50 and adjusting the fraction of DOPC reciprocally. At or below 18 mol % of DOPG, OpuA was already maximally active at  $I_{in} \approx 0.2$  and not much affected when the external KCl concentration was increased from 0 to 0.25, and hence the  $I_{in}$  was raised from  $\approx 0.2$  to  $\approx 0.7$  (Fig. 3 A–C). It is important to emphasize that at  $I_{in} \leq 0.1$ , sigmoidal activation profiles similar to those of Fig. 1 were recorded also with 18 mol % DOPG (data not shown). At or above 25 mol % of DOPG in the membrane, OpuA was inactive when  $I_{in}$  was low. Consistent with previous work (10), the higher the fraction of anionic lipid, the more salt was needed for activation (Fig. 3 D–F).

Contrary to wild-type OpuA, OpuA $\Delta$ 12 was already inhibited at an  $I_{in}$  of  $\approx 0.2$  (0 KCl in Fig. 3 B and C) when the fraction of DOPG was 13 or 18 mol %. At these fractions of anionic lipid,

OpuA $\Delta$ 12 was stimulated by the addition of KCl and hence an increase in  $I_{in}$ . Compared to wild-type OpuA, OpuA $\Delta$ 12 required a higher  $I_{in}$  to reach maximal activity; the open-circle curves (OpuA $\Delta$ 12) in Fig. 3 D–F are shifted to higher salt concentrations relative to the closed-circle curves (OpuA). The activity of OpuA $\Delta$ 61, on the other hand, was  $<2$ -fold different between low and high  $I_{in}$  when DOPG was varied from 6 to 38 mol % (Fig. 3).

The activities of wild-type OpuA, OpuA $\Delta$ 12, and OpuA $\Delta$ 61 as function of the fraction of anionic lipids in the membrane and at low ( $I_{in} \approx 0.2$ ) and high ionic strength ( $I_{in} \approx 0.6$ ) are shown in Fig. 4. The stronger inhibition by anionic lipids of OpuA $\Delta$ 12 is evident at 10–20 mol % of DOPG (Fig. 4B) but, by varying the  $I_{in}$ , the differences between OpuA and OpuA $\Delta$ 12 could be observed over the entire range of anionic lipids (Fig. 3 B–F). OpuA $\Delta$ 61 was largely independent of  $I_{in}$  in the 0.2–0.6 range and displayed a completely different DOPG dependence than wild-type OpuA and OpuA $\Delta$ 12. The residual activation of OpuA $\Delta$ 61 by salt was observed only at high DOPG and may not reflect the genuine electrostatic interaction-based sensing mechanism. The most critical observation is that, contrary to wild-type OpuA and OpuA $\Delta$ 12, OpuA $\Delta$ 61 is no longer inhibited when the fraction of DOPG is high ( $>25\%$ ) and  $I_{in}$  is low ( $<0.2$ ). Clearly, the OpuA $\Delta$ 61 mutant has lost the ionic switching mechanism that becomes operative in OpuA at  $\approx 25\%$  or higher fractions of anionic lipids in the membrane. Although the activity of OpuA $\Delta$ 61 at 35–40 mol % of DOPG was comparable to that of wild-type OpuA, at  $<30$  and  $>45$  mol % of DOPG, the activity was low. The  $I_{in}$  and DOPG dependencies of OpuA $\Delta$ 119 were very similar to those of OpuA $\Delta$ 61 (Fig. 4C *Inset*), except that the measured activities were low because of at least a 10-fold reduced expression of the OpuA $\Delta$ 119 subunit. In our opinion, the moderate salt ( $I_{in}$ ) dependence of OpuA $\Delta$ 61 and OpuA $\Delta$ 119 (Figs. 4C and 3) does not reflect ion sensing to control the transporter activity but some basic ionic strength dependence that any enzyme or transporter may have. Taken together, we propose that the deletion of CBS2 (OpuA $\Delta$ 61) is sufficient to eliminate the ion switch, whereas ATP binding to the ABC subunit and transporter activity (under optimal conditions; Fig. 4C) are comparable to those of wild-type OpuA. The negatively charged C terminus of the CBS shifts the  $I_{in}$  dependence of the transporter to higher values but is not essential for ion sensing.

**Osmotic Activation *in Vivo*.** To assess the physiological relevance of the *in vitro* biochemical experiments, the properties of wild-type OpuA and CBS mutants were also determined in whole cells of *L. lactis*. The initial rate of glycine betaine uptake was measured as a function of external osmolality (Fig. 5; see Fig. 9 A and B, which is published as supporting information on the PNAS web site, and *Supporting Text* on *in vivo* experiments). The activity of wild-type OpuA increased sigmoidally with increasing osmotic stress, i.e., increasing sucrose concentration as shown in Fig. 5, but similar results were obtained with KCl. To activate OpuA $\Delta$ 12, higher concentrations of sucrose (or KCl) were required, whereas OpuA $\Delta$ 61 was largely independent of the applied hyperosmotic stress and displayed only 25% of the maximal activity of wild-type OpuA. Strikingly, these *in vivo* osmotic activation profiles of wild-type OpuA, OpuA $\Delta$ 12, and OpuA $\Delta$ 61 are qualitatively very similar to those of the purified proteins reconstituted in liposomes with 50% DOPG (Fig. 3F). The fraction of anionic lipids in *L. lactis* is  $\approx 60$  mol % and comprises  $\approx 40$  mol % of DOPG derivatives plus  $\approx 20\%$  of glycerophosphoglycolipids (16). The agreement between the two datasets indicates that the *in vitro* measurements are made under physiologically relevant conditions. Also, the osmosensitive phenotype of the *L. lactis* Opu401/pNZOpuA( $\Delta$ 61)His cells *in vivo* (Fig. 9 A and B) is in full agreement with the low activity of OpuA $\Delta$ 61 at  $>45$  mol % of anionic lipid.



residual activation at high DOPG concentrations (see also *Results*). The ionic switch is no longer functional, because tandem pairs of CBS domains, intimately associated by hydrophobic interactions between homologous  $\beta$ -sheets (4, 5), are most probably needed to make a stable structure.

Preliminary experiments indicate that soluble CBS protein indeed physically interacts with the membrane as a function of surface charge and ionic strength (Fig. 10, which is published as supporting information on the PNAS web site). Whether this electrolyte-dependent interaction forms the sole basis for the proposed ionic activation of OpuA needs to be established in future work. On the basis of the published crystal structures, one could predict the regions that would possibly be interacting with negatively charged membrane surfaces. CBS1 has an  $\alpha$ -helical region (denoted H1 in Fig. 7) with cationic residues on the same face of the surface exposed helix. Another potential membrane-interaction site is the turn region (denoted T1) that connects two  $\beta$ -sheets in CBS1 (Fig. 11, which is published as supporting information on the PNAS web site). Irrespective of whether two CBS tandem domains would dimerize, as suggested by the TM0935 structure and confirmed by size-exclusion chromatography experiments with the purified tandem CBS of OpuA (unpublished results), both regions are surface-exposed, as shown from the surface charge distribution of a homology model of the tandem CBS domain (Fig. 11).

**Implications of CBS Domains for Transport Mechanism.** Because both polypeptides of OpuA are present twice in the functional unit, it is possible that the two tandem CBS pairs of the ATPase subunits also form a dimeric structure. In the catalytic cycle of ABC transporters, the nucleotide-binding domains (NBD) come close together (17), and dimerization of two CBS tandems might increase the affinity between the NBDs. This would be reminiscent of the situation in the maltose transporter from *Escherichia coli*, where the accessory regulatory domain linked to the ATPase unit accounts for the high affinity between the NBDs (18). We also emphasize that, unlike most other well studied ABC transporters, *in vitro* ATP hydrolysis and translocation activity are always tightly coupled in OpuA (19). In the presence of glycine betaine but without threshold levels of salt (or reduced levels of anionic lipids), ATP, bound to the NBDs, is not hydrolyzed. Ionic activation may thus involve a repositioning of the NBDs that allows the catalytic cycle to proceed (unlock the system) rather than alter the conformation of the transmembrane domains directly and thereby affect translocation.

Our data show that the C-terminal anionic tail of the CBS domains in OpuA modulates the ion-sensing properties of the transporter. This role in osmosensing is reminiscent to the coiled-coil structure that is present at the C terminus of the osmoregulatory ProP system (20). In ProP, the accessory  $\alpha$ -helical coiled-coil structure is also not essential for osmotic activation of the transporter but tunes the regulation over a specific osmolality range. The sequence of the C-terminal anionic tail of the CBS domains is poorly conserved, and in ProV of the ProU systems from *E. coli* and *Aspergillus fulgidus*, this region is actually missing (Fig. 7). Other homologues of OpuA such as the ProV proteins in methanogenic archaea have extended tails with lengths of more than hundred residues (Fig. 7). The common denominator in many of these sequences are pairs of Glu and/or Asp residues, frequently followed by a Lys residue. We speculate that the C-terminal tails of the CBS domains have evolved to fine tune the ion sensor in response to the abundance of ionic lipids in the membrane and ionic strength of the cytoplasm, which is likely to vary strongly between the different organisms.

**Biological Relevance of Electrostatic Switching Mechanism.** We provide evidence for a relatively simple mechanism of osmosensing, involving protein modules (CBS domains) that are associated

with a wide variety of enzymes and membrane proteins, present in species as diverse as microorganisms, plants, and mammals. The CBS domains of OpuA constitute the sensor that switches the transporter between an inactive electrostatically locked and an active thermally relaxed state by interacting (in)directly with the negatively charged membrane surface in response to the ionic strength. The switching mechanism is an effective means for cells to respond to osmotic shifts, because an increase in medium osmolality will result in a decrease in cell volume, and the accompanying increase in cytoplasmic ionic strength will activate the transporter. Glycine betaine accumulation, followed by water influx, will increase the cell volume and decrease the ionic strength in due time, and the transporter will ultimately be switched off. This inherent feedback mechanism prevents over-accumulation of glycine betaine and couples transporter activity with ionic strength in the cell. Whether other CBS-containing proteins are regulated similarly remains to be determined. It is highly possible that other CBS domains have evolved to sense specific ligands (e.g., adenosine ligands; refs. 8 and 9) rather than ionic strength. Although a high ionic strength activates OpuA (cell shrinkage), the switching logic could be reversed in other CBS-containing proteins, resulting in activation at low ionic strength (e.g., CIC channels activated by cell swelling).

Finally, the high crowding of the cytoplasm and the transient stabilization of macromolecules by screened electrostatic forces suggest that many enzymes and membrane-bound proteins are under the control of ionic strength (21). The CBS domains may be the first of a class of ionic strength sensors to be discovered and, in terms of functional role, it is tempting to make a comparison with the voltage-sensor paddle in the voltage dependent  $K^+$  channels (22). Whereas voltage sensing results from a repositioning of charges in a hydrophobic protein segment within the membrane electric field, ion sensing results from electrostatic interactions between CBS protein segments and the membrane surface. In both cases, structural rearrangements in the sensor are linked to the activation of the transport device. The unraveling of the structural principles associated with the regulation of enzyme and transporter function by CBS domains represents a challenging task for the future.

## Materials and Methods

**Bacterial Strains, Growth Conditions, and Vesicle Preparations.** *L. lactis* strain Opu401 (derivative of NZ9000 in which the *opuA* genes were deleted; see *Supporting Text* for a full description and characterization of the engineered strains) was cultivated semi-anaerobically at 30°C in a medium containing 2% (wt/vol) gistex LS (Strik BV, Eemnes, The Netherlands) and 65 mM Kpi, pH 6.5, supplemented with 1.0% (wt/vol) glucose and 5  $\mu$ g/ml chloramphenicol when carrying pNZopuAHis or derivatives. For the isolation of membrane vesicles, cells were grown in a 2-liter pH-regulated fermentor to an  $A_{600}$  of 2, after which transcription from the *nisA* promoter was switched on by the addition of 0.1% (vol/vol) culture supernatant of the nisin A-producing strain NZ9700. The cells were harvested, and membrane vesicles were prepared according to standard procedures (23).

**Purification and Membrane Reconstitution of OpuA.** OpuA was purified from membrane vesicles by using  $Ni^{2+}$ -NTA chromatography as described (15). The his-tagged proteins were eluted in 50 mM KPi, pH 8.0/200 mM KCl/20% glycerol/0.05% (wt/vol) *n*-dodecyl- $\beta$ -D-maltoside/200 mM imidazole. Purified OpuA and derivatives were reconstituted in liposomes composed of the desired lipids, essentially as described (23), with some modifications (24). The final protein to lipid ratio was 1:100 (wt/wt); synthetic lipids were obtained from Avanti Polar Lipids.



